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Genetic Dissection of the Role of Heparan Sulfate in Mammary Tumor Progression

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13. SUPPLEMENTARY NOTES

14. ABSTRACT
There is accumulating evidence that heparan sulfate (HS) controls various growth factor signaling events. There is also evidence that cellular HS production itself exerts strong influences on tumorigenesis, exemplified by the fact that mutations of *Ext1*, the gene encoding an HS synthesizing enzyme, cause multiple bone tumors. Furthermore, the level of HS degrading activity correlates with the aggressiveness of the tumor. Despite these long-standing observations, much less is known about the mechanisms by which HS influences the malignant behavior of tumors in vivo. Also important is the fact that HS is produced not only by tumor cells themselves but also by stromal cells that constitute the tumor microenvironment. This project will address these key issues by using genetic mouse models. The second year of this project was dedicated to conduct tumorigenesis studies that form the core of the project. Preliminary results suggest that HS indeed affects the progression of mammary tumors. We will continue these experiments during the third year to obtain statistically significant survival data. Analysis of tumors formed in these mice will shed light on the molecular mechanisms by which HS regulates mammary tumor progression.

15. SUBJECT TERMS
Heparan sulfate; Ext1; Tumorigenesis; Tumor microenvironment; Conditional knockout

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Introduction

Heparan sulfate is a linear polysaccharide composed of repeating *N*-acetylglucosamine and glucuronic acid residues. The anticoagulant heparin is a specialized form of heparan sulfate. Heparan sulfate chains are covalently attached to various core proteins to form heparan sulfate proteoglycans (HSPGs). HSPGs exist mainly as cell surface and extracellular matrix molecules and are functionally involved in various biological processes, including growth factor signaling, regulation of morphogen gradient, cell adhesion, lipoprotein metabolism, and modulation of proteinase activities (Gallagher and Lyon, 2000). Considering its interactions with a number of growth factors/cytokines, it is likely that heparan sulfate plays an important role in cancer development and progression. For instance, two signaling molecules that have strong implications in human breast cancer, namely Wnt1 and neuregulin (the Ig-domain containing isoforms), bind and functionally modulated by heparan sulfate. Factors known to affect invasion, metastasis, and tumor angiogenesis, such as matrix metalloproteinases, VEGF, and endostatin, also interact with heparan sulfate. Despite this wealth of data, our understanding of the mechanisms by which heparan sulfate influences tumor cell behavior *in vivo* is still fragmentary. One of the important unknowns is what is the overall physiological effect of heparan sulfate on tumor development and progression. Further compounding the issue is that heparan sulfate is produced not only by tumor cells themselves but also by stromal cells within tumors. The field needs advanced animal models that not only closely mimic clinical cancers but also allow precise dissection of heparan sulfate function in different cell types. The key glycosyltransferase for the biosynthesis of heparan sulfate is the glycosyltransferase called EXT1. EXT1 catalyzes the polymerization of *N*-acetylglucosamine and glucuronic acid residues (Duncan et al., 2001; Zak et al, 2002). Genetic and biochemical studies have established that the *Ext1* gene is absolutely essential for heparan sulfate biosynthesis (Lin et al., 2000). These properties make *Ext1* as an excellent target for genetic disruption of heparan sulfate synthesis. This allows direct interpretation of the causal relationship between heparan sulfate and the resultant phenotype. Our primary objective is to obtain direct information regarding the role of heparan sulfate in breast cancer development and progression in the context of *de novo* mammary tumorigenesis models. An ancillary objective is to determine whether tumor cell-expressed heparan sulfate and stromal cell-expressed heparan sulfate exert distinct effects on the behavior of mammary tumors. We hypothesize that they have different effects on the growth and progression of mammary tumors. Through this project, we will define the role of heparan sulfate in breast cancer under the condition that mimics human breast cancer than ever before, thereby advancing our understanding of the role of heparan sulfate in breast cancer to the next level.

Body

Task 1. Acquisition of animal experiment approval.
Necessary approval for animal experiments was obtained on schedule.

Task. 2. Generation of animal cohorts for studies in Aim 1 (the role of tumor cell autonomous heparan sulfate in mammary tumor development and progression).
As reported in the previous progress report, this part of the project had experienced a problem of low fertility of *KFS2MT6* transgenic mice (Cecena et al., 2006) during the first year, which incurred a delay in this part of the project. This problem had been corrected during the second year by obtaining new breeding pairs from Dr. Robert Oshima, the creator of the transgenic line. We have completed a tumorigenesis study during the third year. As shown in Fig. 1, the ablation of heparan sulfate synthesis in mammary tumor cells themselves (i.e., cell autonomous expression) prolongs the survival of tumor-carrying mice, although eventually these mice die. The behavior of heterozygous tumors was in-between the wild-type and null tumors, suggesting a correlation between the level of heparan sulfate expression and the survival. These results indicate that cell autonomous expression of heparan sulfate plays a role in the progression of tumors during the early phase.

Task 3. Analysis of mammary tumors in Aim 1.
This task had experienced a delay in the previous years, because it depends on tumor samples to be collected from animals used in Task 2. As the tumorigenesis/survival study has been completed, we have now collected tumor samples to be examined in this task. This part of the project will be performed during the forth year under a no-cost extension.

Task 4. Expression profiling of genes between *Ext1* null and control tumors.
By the same reason as stated for Task 3, this task had experienced a delay because of the poor breeding of mice. During the third year, we have accumulated sufficient numbers of tumor samples to be examined. This task will be performed during the forth year under a no-cost extension.

Task 5. Generation of animal cohorts for studies in Aim 2 (the role of stromally produced heparan sulfate in mammary tumor development and progression).
During the second year, we showed that *FSP1-Cre;Ext1*$^{flox/flox}$ mice develop normally, and therefore can be used to study the role of stromal heparan sulfate in tumorigenesis. This task has been completed during the third year. As shown in Fig. 2, we found no difference in survival among wild-type, *PyMT;FSP1-Cre;Ext1*$^{flox/wt}$ (heterozygous), and *PyMT;FSP1-Cre;Ext1*$^{flox/flox}$ (homozygous) mice. This is in contrast to the effect of cell autonomous ablation of heparan sulfate synthesis on tumorigenesis (Fig. 1). These results thus indicate that the

expression of heparan sulfate in stromal cells have little influence on the growth of mammary tumors.

Task 6. Analysis of mammary tumors in Aim 2.

Analysis of lung metastasis. We examined lung metastasis in both *KFS2MT6;MMTV-Cre;Ext1* and *PyMT;FSP1-Cre;Ext1* models during the third year. This study has been largely done, but we need to examine additional animals to complete it. Fig. 3 and Fig. 4 show the preliminary results of these studies. We found that cell autonomous heparan sulfate expression has a significant influence on the number of metastatic lung nodules in the *KFS2MT6;MMTV-Cre;Ext1* model (Fig. 3). Both *KFS2MT6;MMTV-Cre;Ext1*$^{flox/flox}$ (homozygous null) and *KFS2MT6;MMTV-Cre;Ext1*$^{flox/wt}$ (heterozygous) mice developed significantly smaller numbers of metastatic lung nodules than did wild type mice. This indicates that mammary tumor cells with reduced capacity of heparan sulfate synthesis are less metastatic and/or invasive, and suggests that cell autonomous expression of heparan sulfate is an aggravating factor for mammary tumor progression. In the case of the *PyMT;FSP1-Cre;Ext1* model, the number of metastatic nodules was significantly smaller than in *KFS2MT6;MMTV-Cre;Ext1* model, and therefore the results on these two models cannot be compared side-by-side. Yet a similar, but not identical, tendency was found for the *PyMT;FSP1-Cre;Ext1* model. In this case, complete ablation (homozygous deletion of *Ext1*) of heparan sulfate expression in stromal cells resulted in the absence of metastatic nodules in the lung, whereas partial ablation (heterozygous deletion of *Ext1*) had little effects on lung metastasis as in wild-type mice (Fig. 4). Together, although the differences in these analyses are not statistically significant at present, these results seem to indicate that heparan sulfate plays a role in lung metastasis of mammary tumor cells *in vivo*. The effect on metastasis seems to be greater than that on tumorigenesis/survival (see Fig. 1). During the fourth year, we will continue this study to obtain conclusive evidence for the role of heparan sulfate in metastasis of mammary tumors.

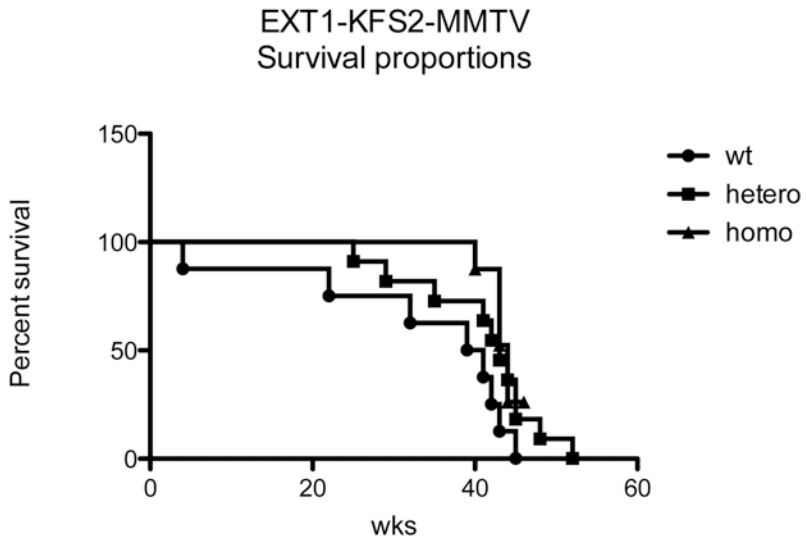

Figure 1. Survival curve of the *KFS2MT6;MMTV-Cre;Ext1* model. (*wt*) wild-type; (*hetero*) *KFS2MT6;MMTV-Cre;Ext1*$^{flox/wt}$; (*homo*) *KFS2MT6;MMTV-Cre;Ext1*$^{flox/flox}$. The number of animals that could eventually be analyzed in this study was 6 (wild-type), 10 (*KFS2MT6;MMTV-Cre;Ext1*$^{flox/wt}$), and 8 (*KFS2MT6;MMTV-Cre;Ext1*$^{flox/flox}$).

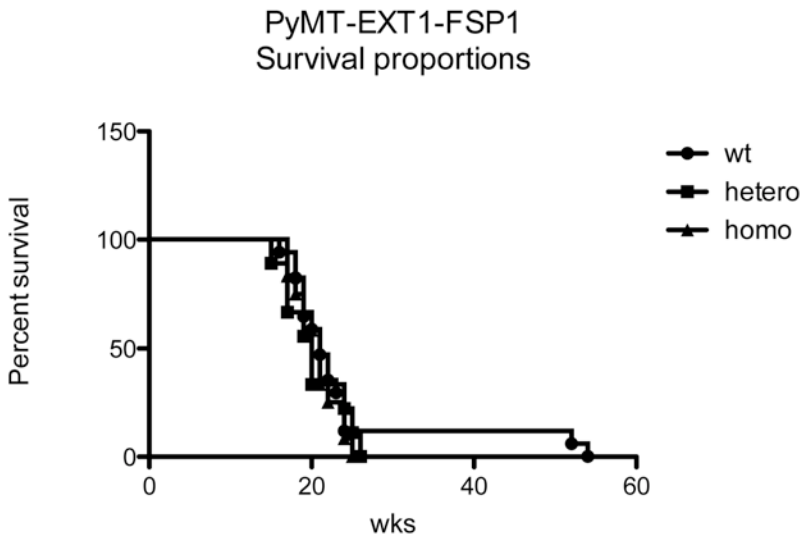

Figure 2. Survival curve of the *PyMT;FSP1-Cre;Ext1* model. (*wt*) wild-type; (*hetero*) *PyMT;FSP1-Cre;Ext1*$^{flox/wt}$; (*homo*) *PyMT;FSP1-Cre;Ext1*$^{flox/flox}$. The number of animals that could eventually be analyzed in this study was 11 (wild-type), 9 (*PyMT;FSP1-Cre;Ext1*$^{flox/wt}$), and 12 (*PyMT;FSP1-Cre;Ext1*$^{flox/flox}$).

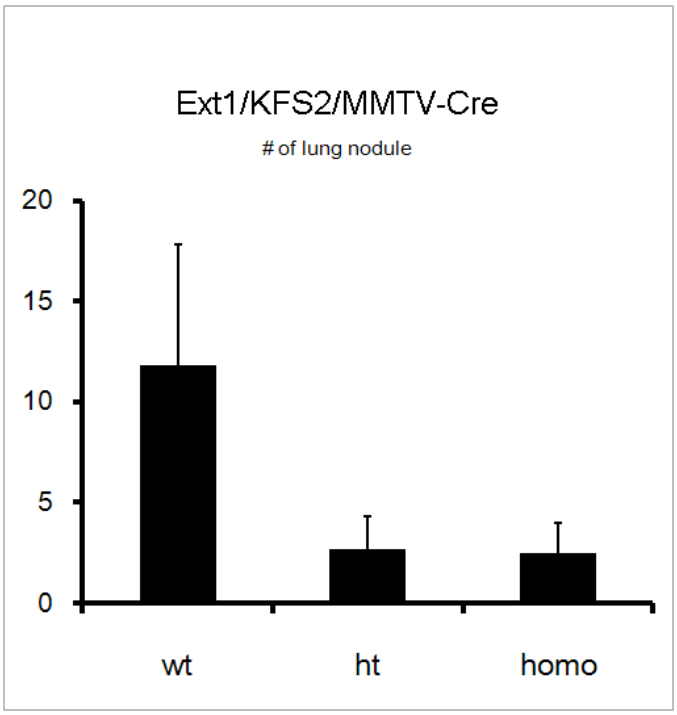

Figure 3. The number of metastatic lung nodules in the *KFS2MT6;MMTV-Cre;Ext1* model.

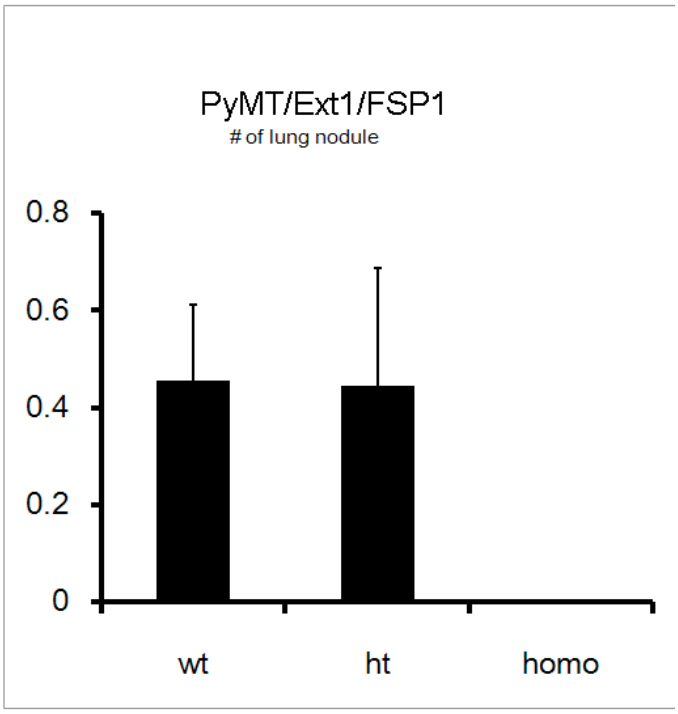

Figure 4. The number of metastatic lung nodules in the *PyMT;FSP1-Cre;Ext1* model.

Key Research Accomplishments

Tumorigenesis/survival study: KFS2MT6;MMTV-Cre;Ext1 model

- This study has been completed during the third year. Results show that the ablation of cell autonomous expression of heparan sulfate inhibits the progression of tumors and the survival of tumor-bearing mice during the early phage.

Tumorigenesis/survival study: PyMT;FSP1-Cre;Ext1 model

- This study has been completed during the third year. Results show that the ablation of heparan sulfate expression in tumor stromal cells does not have a significant influence on the progression of mammary tumors or the survival of tumor-bearing mice.

Study of lung metastasis: KFS2MT6;MMTV-Cre;Ext1 model

- This study has been mostly completed during the third year. Preliminary results show that the ablation of cell autonomous expression of heparan sulfate attenuates lung metastasis.

Study of lung metastasis: PyMT;FSP1-Cre;Ext1 model

- This study has been mostly completed during the third year. Preliminary results show that the ablation of environmental (=stromal) expression of heparan sulfate attenuates lung metastasis.

Reportable Outcomes

N/A

Conclusion

This is the third progress report of this IDEA award. The goal of the project is to obtain physiological evidence for the role of heparan sulfate in mammary tumor development and progression using conditional knockout mouse models. Because of the complexity of the breeding schemes necessary for producing triple compound mutant mice and their control counterparts, the large portion of the grant period was to be spent for crossbreeding of mice. As reported in the previous progress reports, we encountered breeding problems in the first and second years, which incurred some delay in the project and necessitate us to request for a no-cost extension.

Nevertheless, we have been able to complete the core parts of the project by the end of the third year. Most importantly, our results indicate that the ablation of cell autonomous heparan sulfate expression inhibits the progression of mammary tumors *in vivo*. While the effect of ablation on survival is not impressive, the effect on lung metastasis is substantial both in *KFS2MT6;MMTV-Cre;Ext1* and *PyMT;FSP1-Cre;Ext1* models. The lack of substantial effects on survival in *PyMT;FSP1-Cre;Ext1* models is presumably because heparan sulfate autonomously expressed by tumor cells themselves is sufficient to support signaling for tumor growth. The significant effects on metastasis, on the other hand, suggest a differential requirement of heparan sulfate in tumor cell growth and metastasis. Such a differential effect on tumor growth and metastasis has been reported for the *SRC-1* gene (Wang et al., 2008).

In any event, our results have demonstrated that heparan sulfate is functionally involved in the proliferation and metastasis of mammary tumors. The significant effect of heparan sulfate in metastasis may be utilized to develop an anti-metastasis therapy based on the functional intervention of heparan sulfate in tumors. We request a year of no-cost extension to perform the unfinished parts of the project, thereby establishing *in vivo* role of heparan sulfate in mammary tumor progression.

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